Research Article

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Direct effects of iodothyronines on excess fat storage in rat hepatocytes

Elena Grasselli[1], Adriana Voci[1], Laura Canesi[1], Rita De Matteis[2], Fernando Goglia[3], Federica Cioffi[4], Emilia Fugassa[1], Gabriella Gallo[1], Laura Vergani[1,*]

[1]*Dipartimento di Biologia, Università di Genova, Italy;* [2]*Dipartimento di Scienze Biomolecolari, Università di Urbino, Italy;* [3]*Dipartimento di Scienze Biologiche ed Ambientali, Università del Sannio, Benevento, Italy;* [4]*Dipartimento Scienze della Vita, Seconda Università di Napoli, Caserta, Italy*

Background & Aims: Previous studies have demonstrated that 3,5-L-diiodothyronine (T_2) is able to prevent lipid accumulation in the liver of rats fed a high-fat diet. Whether this effect is due to a direct action of T_2 on the liver has not been elucidated. In this study, we investigated the ability of T_2 to reduce the excess lipids in isolated hepatocytes treated with fatty acids (FFAs). The effects of T_2 were compared with those elicited by 3,3′,5-L-triiodothyronine (T_3).

Methods: To mimic the fatty liver condition, primary cultures of rat hepatocytes were overloaded with lipids, by exposure to FFAs ("fatty hepatocytes"), and then treated with T_2 or T_3. Lipid content, morphometry of lipid droplets (LDs), and expression of the adipocyte differentiation-related protein (ADRP) and the peroxisome proliferator-activated receptors (PPAR-α, -γ, -δ) were evaluated. Activities of the lipolytic enzyme acyl CoA oxidase – AOX and the antioxidant enzymes superoxide dismutase – SOD and catalase – CAT were also determined.

Results: FFA-induced lipid accumulation was associated with an increase in both number/size of LDs and expression of *ADRP*, *PPAR-γ*, and *PPAR-δ/β* mRNAs, as well as in the activities of AOX, SOD, and CAT. The addition of T_2 or T_3 to "fatty hepatocytes" resulted in a reduction in: (i) lipid content and LD diameter; (ii) PPAR-γ and PPAR-δ expression; (iii) activities of AOX and antioxidant enzymes.

Conclusions: These data demonstrate, for the first time, a direct action of both T_2 and T_3 in reducing the excess fat in cultured hepatocytes.

Introduction

Excess of triglycerides (TAGs) in the liver results in a pathological condition called non-alcoholic fatty liver disease (NAFLD) or hepatic steatosis [1,2], typically caused by an overflow of fatty acids (FFAs) into the liver [3].

FFAs taken up by the liver are metabolized by esterification to produce TAGs or, alternatively, by oxidation to generate ATP. TAGs are stored under the form of cytosolic lipid droplets (LDs) [4]. Typically, LDs are composed of a core of neutral lipids surrounded by phospholipids and proteins of the PAT protein family. Among them, the adipocyte differentiation-related protein (ADRP, also known as PLIN2) represents the most important protein in the human and mouse liver. ADRP expression is increased in NAFLD [5] and is under the control of peroxisome proliferator-activated receptors (PPARs), transcription factors acting as regulators of lipid metabolism [6,7].

The liver is the target for the thyroid hormones (THs) thyroxine (T_4) and 3,3′,5-L-triiodothyronine (T_3) that play a major role in energy balance and lipid metabolism. THs stimulate fat oxidation, possibly by acting at mitochondrial level [8,9], and their use as potential drugs to treat obesity has been suggested; however, due to their collateral dangerous effects, their employment is now not recommended. The development of TH agonists/analogs that retain lipid-lowering and anti-obesity efficacies while being devoid of thyrotoxic effects would represent a potentially valuable therapeutic advance. Other iodothyronines display some thyromimetic activities. Among them, 3,5-L-diiodothyronine (T_2) mimics several effects of T_3 on energy metabolism without inducing thyrotoxic effects [10]. Indeed, it has been shown that like T_3, also T_2, when injected into hypothyroid rats, is able to stimulate both the resting metabolic rate and mitochondrial activity [11]. Moreover, when administered to rats receiving a high-fat diet (HFD), T_2 is able to both prevent [12,13] and reduce [14] the development of liver steatosis and excessive body weight gain by stimulating FFA oxidation and mitochondrial uncoupling [12–14]. T_2 administration also reduces the oxidative stress conditions induced by excess lipids in the liver [13].

The *in vivo* studies performed so far could not distinguish between the direct antisteatosic effects of THs on the liver and their secondary effects due to upstream changes in endocrine or metabolic pathways. The employment of isolated hepatocytes might help to overcome these problems [15]. Several *in vitro* models of liver steatosis have been developed, mainly consisting of primary hepatocyte cultures [16,17] or hepatocyte cell lines [18] treated with monounsaturated and/or saturated FFAs. These

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* Corresponding author. Address: Dipartimento di Biologia, Corso Europa 26, 16132 Genova, Italy. Tel.: +39 010 3538403.
E-mail address: laura.vergani@unige.it (L. Vergani).

models usually employ palmitate (C16:0) or oleate (C18:1), or a mixture of the two [19], since these are common dietary long-chain FFAs and the most abundant FFAs in the liver of both normal subjects and patients with NAFLD [20].

In the present study, the effects of T_2 and T_3 were assessed *in vitro* using primary cultures of rat hepatocytes overloaded with lipids ("fatty hepatocytes") by exposure to a FFA mixture [5]. The effects of iodothyronines on lipid accumulation in LDs as well as on expression of the PPAR isoforms and ADRP protein were evaluated. Moreover, the activity of acyl-CoA oxidase (AOX), the first enzyme in peroxisomal FFA oxidation, was evaluated, as well as the activities of the antioxidant enzymes superoxide dismutase and catalase. The results demonstrate, for the first time, a direct 'lipid lowering' effect of iodothyronines on cultured hepatocytes.

Materials and methods

Rat hepatocyte culture

Hepatocytes were isolated from adult male Wistar rats (Harlan-Italy, S. Pietro al Natisone, Italy) and cultured as previously described [21]. Animal maintenance and treatment were carried out according to the guidelines of the European Community Council for animal care and use. Twenty-four hours after plating, hepatocytes were incubated with a mixture of oleate/palmitate (2:1 M ratio, final concentration 1.5 mM) for 24 h [5]. Control hepatocytes were incubated in the medium without the addition of FFAs. Afterward, the medium was replaced by fresh D-MEM containing T_2 or T_3 at different concentrations (from 10^{-7} to 10^{-5} M) and cells were incubated for 12–24 h. As a negative control, hepatocytes were cultured with the addition of the vehicle alone. At the end of the treatment, hepatocytes were collected and stored at −80 °C until use. Cell viability was greater than 90% and it was not affected by the treatments. For microscopical analyses, hepatocytes were cultured and treated directly on collagen-coated glass slides (Falcon, BD, Milano, Italy).

Lipid quantification

In intact cells, neutral lipids were visualized using the selective Oil-RedO (ORO) dye [22]. Slides were examined by Nikon Eclipse E80i light microscope (Nikon, Japan). Densitometric analysis was performed with the Optimas 6.5 image analysis system (Optimas, Washington, DC). The triglyceride (TAG) content was quantified using the commercial GPO-PAP kit (Roche, Milano, Italy) [5]. Values were normalized for the protein content determined by the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard [23].

Immunohistochemical staining for ADRP

ADRP was detected on formalin-fixed hepatocytes [24] using polyclonal anti-ADRP antibody (Fitzgerald Industries Int., Concord, MA). The immunoreaction was detected with the ABC peroxidase method (Vector, Burlingame, CA) [25].

RNA extraction and real-time RT-PCR

Total RNA was isolated using the Trizol reagent (Sigma–Aldrich Corp. Milano, Italy) [26]. First strand cDNA was synthesized from 1 μg of RNA [5]. The gene expression levels were quantified by quantitative RT-PCR (q-PCR) using Chromo4™ System PCR apparatus and iTaq SYBR Green Supermix (Biorad, Milano, Italy) [27]. *PPAR-δ* primers (FWD: 5′-AATGCCTACCTGAAAAACTTCAAC-3′ and REV: 5′-TGCCTGCCACAGCGTCTCAAT-3′) were designed *ad hoc* starting from the coding sequences of *Rattus norvegicus*. All other primers employed in this study have been described elsewhere [5,13]. The relative quantity of target mRNA was calculated by using the comparative C_T method, normalized for the expression of *GAPDH* and expressed as fold induction with respect to controls [28].

Determination of enzyme activities

CAT activity was evaluated in both 12,000×*g* of supernatant and pellet of hepatocyte lysates [29], following the consumption of H_2O_2 at 240 nm. Total CAT specific activity (as the sum of both pellet and supernatant) was expressed as micromoles of decomposed H_2O_2 per min/mg of sample protein. Both SOD and AOX activities were evaluated in the 12,000×*g* supernatant fraction. Cytosolic SOD activity was evaluated as the inhibition of the reduction rate of cytochrome *c* by O_2^- [30] and specific activity was expressed as mU/mg of the sample protein. AOX activity was calculated following the H_2O_2 decomposition coupled to the oxidation of dichlorofluorescein-diacetate [31] and specific activity was expressed as nanomoles of decomposed H_2O_2 per min/mg of sample protein. Spectrophotometric analyses were carried out at 25 °C. Protein content was determined by BCA method [23].

Statistics

Data on both q-PCR and enzyme activities are expressed as means ± SD of at least four independent experiments performed in triplicate. Statistical analysis was performed by using ANOVA followed by Bonferroni *post hoc* test.

Results

Effects of lipid accumulation in primary cultures of rat hepatocytes

Primary hepatocytes were exposed to a mixture of oleate/palmitate to obtain the "fatty hepatocytes". The time-course of FFA exposure for 12–36 h, showing the progressive lipid accumulation and excluding any toxicity of the FFA mixture, has been previously reported [5].

In control hepatocytes, the ORO staining revealed the presence of small LDs (Fig. 1A). Incubation with FFAs for 24 h (FFAs) resulted in a marked lipid accumulation (Fig. 1A) and densitometric analysis estimated an increase in neutral lipid content of +197% with respect to controls (p <0.001) (Fig. 1B). An increase of +93% (p <0.001) in TAG content was observed in "fatty hepatocytes" (Fig. 1C).

Possible changes in size of LDs in "fatty hepatocytes" were analyzed by ADRP immunostaining (Fig. 2A). In control hepatocytes, a few small ADRP-positive droplets (diameters 0.6–3.3 μm) were present dispersed in the cytosol (microvesicular LDs). In "fatty hepatocytes", the cytoplasm was filled up with small ADRP-positive droplets; moreover, we observed the appearance of large droplets with diameters ranging from 5.3 to 20 μm (macrovesicular LDs). In "fatty hepatocytes", macrovesicular LDs (10–15 droplets/cell), were apparently distributed throughout the cytosol without a clear indication of location. Densitometric analysis (Fig. 2B) showed a significant increase in ADRP staining in "fatty hepatocytes" with respect to controls (+118%; p <0.001), this result was confirmed at the mRNA level (Fig. 2C) with a significant up-regulation of ADRP expression in "fatty hepatocytes" (1.4-fold induction; p <0.001).

Lipid excess in isolated hepatocytes is usually associated with up-regulation of PPAR expression [5]. Fig. 3 reports a significant increase in *PPAR-γ* (Fig. 3A) and *PPAR-δ* (Fig. 3B) mRNA level in "fatty hepatocytes" (about 1.7-fold induction compared to controls for both isoforms; p <0.001 for *PPAR-γ* and p <0.05 for *PPAR-δ*), whereas *PPAR-α* expression did not change significantly (Fig. 3C).

At shorter times of FFA treatment (12 h), a significant increase in AOX activity was observed (+29% compared to control; p <0.001), whereas only a slight and not significant increase was observed at 24 h (Fig. 4A). In parallel, the activity of SOD and CAT increased in "fatty hepatocytes" (Fig. 4B and C). These increases were larger after exposure to FFAs for 12 h than for 24 h (+110%, p <0.001 for SOD and +107%, p <0.001 for CAT at 12 h;+29%, p <0.05 for SOD and +61%, p <0.05 for CAT at 24 h).